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Mutational Analysis of the *sbo-alb* Locus of *Bacillus subtilis*: Identification of Genes Required for Subtilosin Production and Immunity

GUOLU ZHENG,[1] ROBIN HEHN,[1,2] AND PETER ZUBER[1]*

Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, Beaverton, Oregon 97006,[1] and Portland Lutheran High School, Portland, Oregon 97233[2]

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The *Bacillus subtilis* 168 derivative JH642 produces a bacteriocin, subtilosin, which possesses activity against *Listeria monocytogenes*. Inspection of the amino acid sequence of the presubtilosin polypeptide encoded by the gene *sboA* and sequence data from analysis of mature subtilosin indicate that the precursor subtilosin peptide undergoes several unique and unusual chemical modifications during its maturation process. The genes of the *sbo-alb* operon are believed to function in the synthesis and maturation of subtilosin. Nonpolar mutations introduced into each of the *alb* genes resulted in loss or reduction of subtilosin production. *sboA*, *albA*, and *albF* mutants showed no antilisterial activity, indicating that the products of these genes are critical for the production of active subtilosin. Mutations in *albB*, *-C*, and *-D* resulted in reduction of antilisterial activity and decreased immunity to subtilosin, particularly under anaerobic conditions. A new gene, *sboX*, encoding another bacteriocin-like product was discovered residing in a sequence overlapping the coding region of *sboA*. Construction of an *sboX-lacZ* translational fusion and analysis of its expression indicate that *sboX* is induced in stationary phase of anaerobic cultures of JH642. An in-frame deletion of the *sboX* coding sequence did not affect the antilisterial activity or production of or immunity to subtilosin. The results of this investigation show that the *sbo-alb* genes are required for the mechanisms of subtilosin synthesis and immunity.

The bacteriocins are a group of antimicrobial peptides that are produced by microorganisms inhabiting diverse environments (10, 12, 14). Typically, these small, gene-encoded polypeptides are first made as unprocessed, unmodified precursors that often undergo interesting and unique chemical modifications during their maturation. They possess hydrophobic N termini that mediate secretion and are subsequently removed proteolytically, yielding the active bacteriocins. The lantibiotic class of bacteriocins, the so-called group I bacteriocins (7, 10, 12, 44), include modified polypeptides that contain dehydrated threonine (T) or serine (S) residues, which condense with cysteine (C) to yield methyllanthionines and lanthionines. The lanthionines form intrachain thioether bridges that impart a cyclic character to the group I bacteriocins. Lantibiotics are produced by gram-positive bacteria such as the *Bacillus* species, the lactic acid bacteria (*Lactococcus*, *Lactobacillus*, and *Carnobacterium* spp.), and other gram-positive cocci (7, 10, 12, 34, 51). Since many of these bacteria are common components of fermented food, they have found use in food preservation and the inhibition of food-borne pathogens (18, 19). The lantibiotics, such as nisin, and the unmodified, or group II, bacteriocin pediocin PA-1 are used to reduce bacterial contamination in dairy products and meats (19).

The spore-forming, nonpathogenic soil bacterium *Bacillus subtilis* is capable of growth under aerobic and anaerobic conditions (24, 39) on a variety of substrates and is a common component of traditional fermented foods (2), particularly in Asian and African cultures. It also is known to produce an abundance of antimicrobial compounds, including the bacteriocin subtilosin (1, 49). While many lantibiotics and group II bacteriocins have been discovered and characterized (19), subtilosin is unique in that it appears to undergo unusual chemical modifications during its maturation that are unlike those of lantibiotics. Although codons for Phe are present in the nucleotide sequence of the gene (*sboA*) encoding subtilosin, no Phe residues are detected by amino acid analysis or by sequencing of peptides released from purified subtilosin through partial hydrolysis or proteolysis (1, 49). Other unusual properties can be discerned by inspection of the amino acid sequence, particularly the very short leader peptide of the subtilosin precursor and the covalent linkage of the N-terminal Asn and the C-terminal Gly.

We had reported that the operon was composed of eight genes (*sboA* and *albABCDEFG*). It is likely that some of the products of the *alb* genes function in carrying out the unusual modifications and processing of the subtilosin prepeptide. A search of proteins showing homology with the amino acid sequence of the *alb* products reveals similarities to proteins that are known to be involved with peptide processing, some of which are unlike those that carry out the processing of lantibiotics. Knowledge of how modifications are carried out during subtilosin maturation may provide new strategies for chemically modifying peptides.

The transcriptional start site of *sbo-alb* was localized to a sequence at position −45 with respect to the ATG translational start codon of *sboA*. It is preceded by a −35 and −10 region resembling a σ^A-utilized promoter. A stem-loop structure located at the end of the *sboA* gene and upstream of the *albA* coding sequence may reduce transcriptional readthrough into the *alb* genes, thereby ensuring that the *alb* products are produced in smaller amounts with respect to the SboA substrate peptide. The *sbo-alb* genes are transcriptionally regulated by factors, such as Spo0A (8) and AbrB (41), which control the nutritional stress response and processes of cellular differentiation in *B. subtilis*. In a previous report (T. Stein, S.

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, 20000 NW Walker Rd., Beaverton, OR 97006. Phone: (503) 748-7335. Fax: (503) 748-1464. E-mail: pzuber@bmb.ogi.edu.

TABLE 1. Strain used in this study

Strain	Genotype	Reference or source
JH642	*trpC2 pheA1*	J. A. Hoch
ORB3148	*trpC2 pheA1 sbo*::*neo-1*	49
ORB3149	*trpC2 pheA1 sbo*::*neo-2*	49
ORB3351	*trpC2 pheA1 albA*::pAG-albA (Cm^r $Phle^r$)	This study
ORB3400	*trpC2 pheA1 albB*::*cat* (Cm^r)	This study
ORB3353	*trpC2 pheA1 albC*::pAG-albC (Cm^r $Phle^r$)	This study
ORB3354	*trpC2 pheA1 albD*::pAG-albD (Cm^r $Phle^r$)	This study
ORB3355	*trpC2 pheA1 albE*::pAG-albE (Cm^r $Phle^r$)	This study
ORB3356	*trpC2 pheA1 albF*::pAG-albF (Cm^r $Phle^r$)	This study
ORB3354	*trpC2 pheA1 albG*::pAG-albG (Cm^r $Phle^r$)	This study
ORB3412	*trpC2 pheA1 amyE*::pTRP-sboX (Cm^r)	This study
ORB3413	*trpC2 pheA1 amyE*::pTRP-sboX *sboA*::neo (Cm^r Neo^r)	This study
ORB3441	*trpC2 pheA1 amyE*::pTRP-sboX *abrB*::*neo* (Cm^r Neo^r)	This study
ORB3442	*trpC2 pheA1 sboX*::*neo-1* (Neo^r)	This study
ORB3445	*trpC2 pheA1 sboX*::*neo-2* (Neo^r)	This study
ORB3470	*trpC2 pheA1 amyE*::pDR-sboAXalbABC′ (Cm^r)	This study
ORB3471	*trpC2 pheA1 amyE*::pDR-sboAXalbABC′::*spc* ($Spec^r$)	This study
ORB3472	*trpC2 pheA1 amyE*::pDR-sboAXalbABC′::*spc albB*::*cat* ($Spec^r$ Cm^r)	This study
ORB2552	*pheA1 sboX*::*neo-1*	This study
ORB3568	*pheA1 sboXΔ89-139*	This study

Düsterhus, A. Stroh, and K.-D. Entian, Abstr. 10th Int. Conf. Bacilli, abstr. P103, p. 65, 1999) and in the accompanying paper (23), we show that *sbo-alb* is induced under anaerobic conditions and controlled by the ResDE signal transduction system that regulates gene expression in response to limiting oxygen (24, 26, 42).

In this report, the phenotypes caused by nonpolar insertion mutations in each of the *alb* genes are described along with the identification of a new gene, *sboX*, the coding sequence of which overlaps the *sboA* gene. Genes required for immunity to subtilosin are also identified.

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotide primers. The bacterial strains used in this study are described in Table 1. All are derivatives of *B. subtilis* strain JH642. The indicator bacterium *Listeria monocytogenes* F4244, which was used to detect production of subtilosin, was obtained from M. Slavik (48). Strains ORB3148 and ORB3149 bear *neo* gene cassette insertions in the *sboA* gene (49). In ORB3148, the *neo* gene is oriented in the direction of *sbo-alb* transcription, and in ORB3149, the *neo* gene is oriented in the opposite direction.

The oligonucleotides used in this study are described in Table 2. Plasmids pAG-albA, pAG-albC, pAG-albD, pAG-albE, pAG-albF, and pAG-albG are derivatives of pAG58-ble-1 (47) and were used to create nonpolar disruptions of the *alb* genes. To construct these plasmids, a *Hin*dIII-*Xba*I fragment containing an internal segment of an *alb* gene (except *albB*) was generated by PCR using a

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence
oGZ1	CAGCTCAAGCTTACCAAATGACATTTT
sboP1	CCTCATGACCAGGACTTCGCCTTCGCTTACTTT
sboP2	CGGTGCCGAGCGCTTCAGGTAAGCTTTCCAAA
sboP3	TGCTGGATCCGAGCCGCTTGTCTAGTGGACGGTCCTAT
sboP4	CTCGGATCCAGCATGTTGCACAACCTTTGTTTTCTA
oywiA-U	GAGCAGAAGCTTCCATTTATTAATGAAA
oywiA-L	CACTATCTAGAGAAATGCCGACGACGATGATTT
oywiQ-U	GGGACAACGTAAGCTTAGAACAAGTGGATT
oywiQ-L	GCACGACATCTAGAAGATGAGAGAAGAAAA
oywhP-U	GCGCAAAGCTTATATCATACTCGCGCTTCTT
oywhP-L	GGTGTTCATCATTCTAGAACGAAAGAA
oywhO-U	GCCGCAAGCTTTCTGAAACCGATCGAAA
oywhO-L	CCCCATCTAGACCAATAGACCTTCGGGAGAAT
oywhN-U	GGCCGCAAGCTTTACAGTGATTTTTTT
oywhN-L	GAGAGGTCTAGATTTCATGCCGGGATGATT
oywhM-U	GCACTGTCTTTAAGCTTTTATTGCTGCTTA
oywhM-L2	CACTTTTCTAGACAGGCTGCCGTGCCGGAAAA
oywhR-U1	GTGGGAATTCCACGATATGGCCCAAAA
oywhR-L1	CCAAGGAAAGCTTCGAAATTCTCCTATT
oorfX-U	GCGGAATTCGATGACTTCTTGGTTT
oorfX-L	GGGAGAAGCTTACCCCCATAGACCGAATA
oorfX-U1	CGCGGATCCCCCAAAAGGGCATAGTCATT
oorfX-L1	CGCGGATCCTTACCCCCATAGACCGAATA
osboXd-3	TTAAGGATCCCGTTTTTCATTTGAATCATAT
osboXd-5	AACGGGATCCTTAAAAAGGGCATAGTA

pair of oligonucleotides as detailed below. Each individual fragment was then inserted into *Hin*dIII-*Xba*I-cleaved pAG58-ble-1. The resulting plasmids, pAG-albA, pAG-albC, pAG-albD, pAG-albE, pAG-albF, and pAG-albG, were used to transform competent cells of JH642 with selection for chloramphenicol resistance (Cm^r) to create *alb* gene disruption mutants. To construct pAG-albA, oligonucleotides oywiA-U and oywiA-L were used in a PCR to obtain a fragment extending from 9 to 681 bp downstream of the TTG translation start site of *albA*. For pAG-albC, oligonucleotides oywiQ-U and oywiQ-L were used to generate a PCR fragment extending from 43 to 604 bp downstream of the ATG start site of *albC*. In the construction of pAG-albD, oligonucleotides oywhP-U and oywhP-L were used to generate a PCR fragment extending from 155 to 786 bp downstream of the ATG start site of *albD*. To construct pAG-albE, oligonucleotides oywhO-U and oywhO-L were used to generate a PCR product containing sequences from 49 to 1,035 bp downstream of the TTG start site of *albE*. In the construction of pAG-albF, oligonucleotides oywhN-U and oywhN-L were used to generate a PCR fragment extending from 219 to 725 bp downstream of the ATG start site of *albF*. To create pAG-albG, oligonucleotides oywhM-U and oywhM-L2 were used to generate a PCR fragment extending from 10 to 546 bp downstream of the ATG start site of *albG*.

To construct pUC-albB::CAT, an *Eco*RI-*Hin*dIII fragment containing the *albB* gene along with the sequence 547 bp upstream and 577 bp downstream of *albB* was generated by PCR using the oligonucleotides oywihR-U1 and oywihR-L1. The *Eco*RI-*Hin*dIII fragment was inserted into *Eco*RI-*Hin*dIII-cleaved pUC18 to create pUC-albB. Plasmid pUC-albB was cut with *Bss*HII at the two *Bss*HII sites within the *albB* gene and treated with T4 DNA polymerase (28) to create blunt ends. A *Sma*I fragment bearing a *cat* (Cm^r) cassette from pMMN7 (25) was inserted into the blunt ends of pUC-albB to create pUC-albB::CAT. The orientation of the *cat* gene in the plasmid was determined by restriction analysis. The resulting pUC-albB::CAT plasmid was used to transform competent cells of JH642 to obtain the *albB*::CAT insertion mutant, the structure of which was confirmed by PCR using oligonucleotides oywhR-U1 and oywhR-L1.

The *sboX* translational *lacZ* fusion was constructed by using plasmid ptrpBGI (37), which bears a promoterless *lacZ* gene. An *Eco*RI-*Hin*dIII fragment extending from 477 bp upstream to the 14th codon downstream of the putative TTG start site of *sboX* was generated by PCR using primers oorfX-U and oorfX-L. The PCR product was inserted into *Eco*RI-*Hin*dIII-cleaved ptrpBGI, which placed the *sboX* coding sequence in frame with the *lacZ* coding sequence. The resulting plasmid, pTRP-sboX, was used to transform competent cells of JH642 and ORB3148 to generate ORB3412 and ORB3413, respectively. The amylase-defective phenotype of ORB3412 and ORB3413 was confirmed by the iodine staining method (28). The chromosomal DNA of LAB2332 was used to transform competent cells of ORB3412 with selection for Cm^r and Neo^r to obtain ORB3441.

Plasmid pUC-sboXBH is a derivative of pUC18 (47). A *Bam*HI-*Hin*dIII fragment extending from 45 to 490 bp downstream of the TTG start codon of *sboX* was generated by PCR using oligonucleotides oorfX-U1 and osboP2 and inserted into *Bam*HI-*Hin*dIII-cleaved pUC18. An *Eco*RI-*Bam*HI fragment extending from 477 bp upstream to 45 bp downstream of the TTG start site of *sboX* was generated by PCR using primers oorfX-U and oorfX-L1 and cloned into the *Eco*RI-*Bam*HI sites of pUC-sboXBH. The resulted plasmid was pUC-sboXEBH. To construct an *sboX*::*neo* strain, a *Bgl*II-*Bam*HI fragment bearing the *neo* cassette from pDG782 (6) was inserted into the *Bam*HI site of pUC-sboXEBH and the orientation of the *neo* cassette was determined by restriction analysis. The pUC-sboXEBH derivative containing the *neo* cassette was used to transform competent cells of JH642 to obtain strain ORB3442. The *sboX*::*neo* insertion mutation was confirmed by PCR using osboP2 and osboP3.

To make pDR-sboAXalbABC′, a *Hin*dIII-*Cla*I fragment extending from 490 bp upstream of the start codon of *sboA* to 510 bp downstream of the start codon of *albC* was made by PCR using the primers oGZ1 and oywiQ-L. The fragment was then inserted into *Hin*dIII-*Cla*I-cleaved pDR67, thus creating plasmid pDR-sboAxalbABC′. pDR-sboAxalbABC′ was used to transform cells of JH642 with selection for Cm^r and screening for *amyE* (amylase negative) to obtain ORB3470. The Cm^r marker of ORB3470 was replaced with $Spec^r$ by using plasmid pCm::Sp (40) to obtain strain ORB3471. The chromosomal DNA of ORB3471 was then used to transform cells of *albB* mutant strain ORB3400 to obtain ORB3472.

Plasmid pUC-sboXEBH-D was constructed to create an in-frame deletion within the *sboX* coding sequence. A *Bam*HI-*Hin*dIII fragment containing 138 to 490 bp downstream of the TTG start site of *sboX* was generated by PCR using oligonucleotides osboXd-3 and osboP-2 and inserted into *Bam*HI-*Hin*dIII-cleaved pUC18 to generate pUC-sboXBH-D. *Eco*RI-*Bam*HI-cleaved pUC-sboXBH-D was ligated to an *Eco*RI-*Bam*HI fragment extending from 477 bp upstream to 89 bp downstream of the TTG start site of *sboX* which had previously been obtained by PCR using primers osboXd-5 and osboP-1. The resulting plasmid, pUC-sboXEBH-D, and chromosomal DNA of ORB3552 were then used to transform cells of strain ORB3442 with selection for Trp^+ and screening for neomycin sensitivity. The *sboXΔ89-138* in-frame deletion in ORB3568 thus created was confirmed by PCR and DNA sequence analysis.

Culture media. 2×YT broth (22) was used for the routine growth of *Escherichia coli* and *B. subtilis*. A yeast extract-glucose (YG) agar previously described (49) was used to grow JH642 or ORB3148 for analysis of the sensitivity of *alb* mutants toward subtilisin A. YG broth with addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (or chloramphenicol at 5 μg/ml for the *albB* mutant) was used for examination of the subtilisin A production of *alb* mutants. 2×YT broth supplemented with 1% glucose and 0.2% KNO_3 was used for the growth of *B. subtilis* in the β-galactosidase activity assays and the immunity analysis using partially purified subtilosin A. Brain heart infusion medium was used for growth and maintenance of *L. monocytogenes* F4244.

Transformation and transduction. The preparation of competent *E. coli* and *B. subtilis* cells and genetic transformation were carried out as previously described (4, 22).

β-Galactosidase assay. *B. subtilis* cells were cultured in 2×YT broth supplemented with 1% glucose and 0.2% KNO_3 under aerobic or anaerobic conditions as previously described (20). Samples were withdrawn at 1-h intervals for measurement of β-galactosidase activity (50).

Bioautography of subtilosin by SDS-PAGE. The purity and activity of subtilosin samples from the wild type and *alb* mutants were analyzed by sodium dodecyl sulfate (SDS)–16.5% polyacrylamide gel electrophoresis (PAGE) with Tricine running buffer (36). Each sample (2 × 10 μl) was applied to duplicate gels. After electrophoresis at 100 V for 2 h, each lane was cut vertically from the gels. For each sample, one gel slice was stained by Coomassie blue staining solution and the other was assayed for inhibitory activity by bioautography using the indicator strain *L. monocytogenes* F4244 as described by Zheng and Slavik (48).

Partial purification of subtilosin A. Subtilosin A purification was carried out as previously described (49), with the following modifications. Subtilosin A was precipitated from culture fluid by ammonium sulfate to 80% saturation, extracted for 1 h by 1/20 of the culture volume of methanol, concentrated by evaporation at 55°C, and then resolved by LH-20 chromatography. Subtilosin A fractions were dried by evaporation and dissolved in 20 mM Tris-HCl buffer (pH 7.0). This subtilosin A preparation was electrophoretically pure as demonstrated by Tricine SDS-PAGE. The subtilosin A concentration was determined spectrophotometrically by using the Protein Assay (Bio-Rad, Hercules, Calif.) with bovine serum albumin as the standard.

Immunity assay. Immunity of *alb* mutants was examined by two methods. Cells of JH642 and the *alb* mutants were grown in 2 ml of 2×YT broth (for *alb* mutants chloramphenicol at 5 μg/ml was added). Cells were collected at exponential phase (optical density at 600 nm [OD_{600}], 0.6 to 0.9), and the OD_{600} was adjusted to 0.5 by adding 2×YT broth. In the first immunity assay, 50-μl volumes of these suspensions were mixed with 5 ml of soft 2×YT (0.8% agar) and then poured onto YG plates onto which JH642 and ORB3148 had previously been stabbed and incubated at 37°C for 24 h. The overlaid plates were incubated for another 18 to 24 h at 37°C under aerobic and anaerobic conditions to observe the appearance of inhibition zones around JH642 or ORB3148 colonies. In the second immunity analysis, 50-μl volumes of the 2×YT suspensions were blended with 5-ml volumes of soft 2×YT (0.8% agar supplemented with 1% glucose and 0.2% KNO_3) and then poured onto 2×YT agar (supplemented with 1% glucose and 0.2% KNO_3). For the *albB* mutant, the soft agar contained chloramphenicol at 5 μg/ml. For all other *alb* mutants, the soft agar included 1 mM IPTG. Duplicate plates were made for each mutant and dried for 30 min in a 37°C incubator. Ten-microliter volumes of serial twofold dilutions of subtilosin (>90% pure) were spotted onto the surfaces of the plates, which were then incubated aerobically or anaerobically at 37°C overnight to observe the appearance of inhibition zones. The minimum concentration of subtilosin A that resulted in a clear inhibition zone on each plate was determined and reported as the MIC (see Table 3).

RESULTS

The *albA* and *albF* products are critical for the production of subtilosin. The *sbo-alb* genes are organized in an operon of 7 kb and are transcribed from a promoter residing upstream of the subtilosin structural gene *sboA* (Fig. 1). Nonpolar insertion mutations were created in each of the seven *alb* genes to determine if each functions in the production of and immunity to subtilosin. For *albA*, *-C*, *-D*, *-E*, *-F*, and *-G*, an internal fragment of the N-terminal coding half of each gene was amplified by PCR. Each fragment was inserted into the plasmid pAG58-bleo-1. Integration of the resulting recombinant plasmids into the *alb* locus by homologous recombination yielded insertion mutations that disrupt the *alb* open reading frames (Fig. 1). The Pspac promoter of pAG58-bleo-1 is directed downstream of the plasmid insertion so as to eliminate potential polarity effects exerted by the integrated plasmid. The *albB* gene is too small to create a gene disruption using an internal fragment of the *albB* coding sequence. In this case, a *cat* gene cassette was inserted into the *Bss*HII sites residing within the *albB* gene and the resulting construct was introduced by double recombination into the *sbo-alb* operon. We had observed pre-

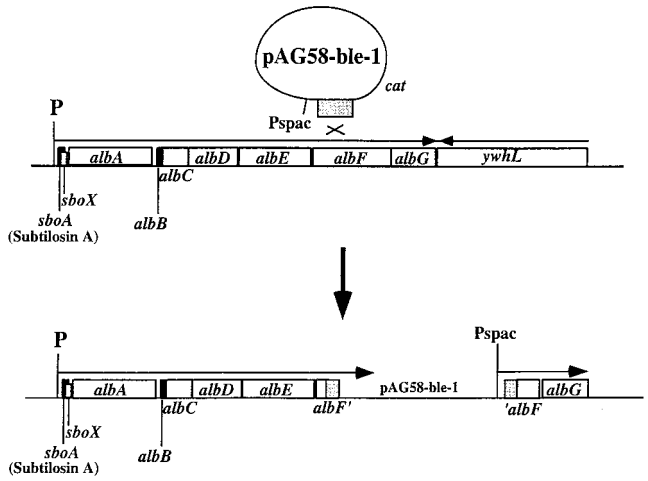


FIG. 1. Organization of the *sbo-alb* operon and the recombination between the integrative plasmid and *alb* DNA that gives rise to the nonpolar insertion mutation. The example shown is *albF*. An internal region of the *albF* gene is amplified by PCR and inserted into the integration vector pAG58-bleo-1. After recombination, a copy of the plasmid is integrated into the *alb* locus. *albF* is disrupted, and transcription from the *sbo* promoter is blocked by the plasmid DNA. Expression of the downstream genes is driven by the IPTG-inducible Pspac promoter.

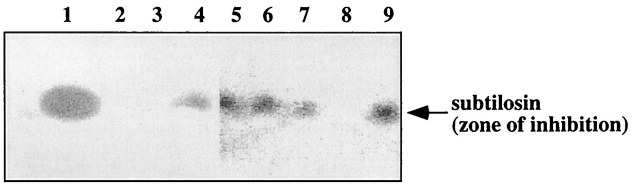


FIG. 2. Bioautography of extracts from culture fluid of *B. subtilis* strain JH642 and the *sboA* and *alb* mutants. Supernatant fluid from YG liquid batch cultures collected at 36 h was subjected to $(NH_4)SO_4$ precipitation, followed by methanol extraction. Methanol extracts were evaporated, and the residue was dissolved in 20 mM Tris-HCl buffer, pH 7.0 (see Materials and Methods). Samples from extracts of wild-type and mutant culture supernatants were applied to Tricine SDS-polyacrylamide gels, and bioautography was performed. A zone of inhibition is observed in the area of the *L. monocytogenes* overlay corresponding to the position of bacteriocin in the gel (indicated by arrow). Lanes: 1, JH642; 2, *sboA*::*neo*; 3, *albA*; 4, *albB*; 5, *albC*; 6, *albD*; 7, *albE*; 8, *albF*; 9, *albG*.

viously that the *cat* gene, if oriented in the same direction as the transcription of the operon into which it is inserted, directs transcription through the genes downstream of the insertion (21).

Each of the mutations thus generated was examined for effects on subtilosin production and immunity (Table 3). Subtilosin production was tested using the critical-dilution assay and bioautography (Fig. 2) of supernatants of YG cultures incubated for 36 h. The wild-type parent JH642 showed antilisterial activity and produced subtilosin, but each of the *alb* insertion mutants produced either none or reduced amounts of the bacteriocin. Only the *sboA*::*neo-1*, *albA*, and *albF* mutants failed to produce any detectable subtilosin. The *albB*, *-C*, *-D*, *-E*, and *-G* mutants exhibited antilisterial activity and produced small amounts of subtilosin, as judged by SDS-PAGE and bioautography (Fig. 2).

The *albB*, *albC*, and *albD* products function in subtilosin immunity. A bacteriocin-producing bacterium is immune to the specific bacteriocin that it produces (13, 35). The genes encoding immunity proteins are usually situated within the operon containing the genes required for bacteriocin biosynthesis. Products that function in immunity have been identified as small membrane-associated peptides, some having a lipid modification that is thought to anchor the peptide to the cytoplasmic membrane (33, 35). The *sbo-alb* operon contained one gene, *albB*, that encodes a small hydrophobic protein that we felt might function in subtilosin immunity. We tested all of the *alb* insertion mutants for defects in immunity by overlaying a lawn of *alb* mutant cells on colonies of JH642 or the *sboA*::*neo-1* mutant and observing the zone of inhibition created (data not shown) or by determining the MIC of subtilosin required to create an inhibition zone on lawns of *alb* mutant cells embedded in a soft agar overlay (Table 3). Only the *albB*, *-C*, and *-D* mutants showed a reduction in subtilosin immunity, with the latter exhibiting the mildest defect.

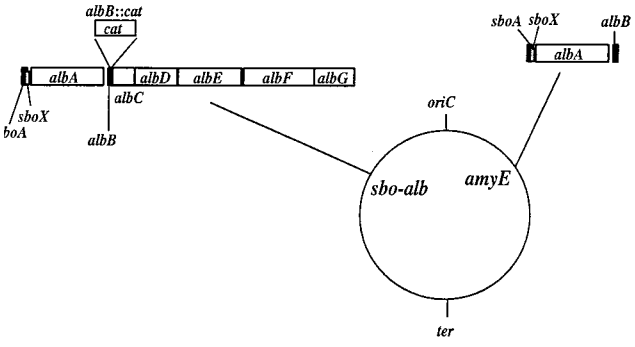
Although the *cat* gene used to create the *albB* insertion mutation had been shown to exert little polarity, a complementation experiment was designed to further show that the product of *albB* is necessary for optimal immunity to subtilosin. A DNA fragment containing the *sbo-alb* promoter, *sboA*, *sboX*, *albA*, and *albB* was inserted into the *amyE* integration plasmid pDR67. A partial diploid strain was constructed that contained the *albB*::*cat* insertion and the integrated *sboAXalbABC'* construct (Fig. 3A). In the absence of added chloramphenicol, the ectopic *albB* allele only partially complemented the *albB* mutation (Fig. 3B). Addition of inducing levels of chloramphenicol, which activates expression of the *cat* gene insertion that

TABLE 3. The *sbo* and *alb* genes and the phenotypes conferred by their mutant alleles

Gene	Product name or length (molecular mass)[a]	Homolog(s) (function)	Mutant phenotype	
			Antilisterial activity (%)[b]	Immunity (MIC [μg/ml])[c]
sboA	Subtilosin	Bacteriocin	− (<0.5)	+ (280)[d]
albA	449 (51,493)	NirJ, PqqE, MoeA (cofactor synthesis)	− (<0.5)	+ (280)
albB	53 (6,116)	? (immunity)	± (6.5)	− (63)
albC	240 (27,232)	ABC transporter, ATP-binding protein	± (12.5)	− (96)
albD	436 (49,516)	?	± (6.5)	− (150)
albE	399 (44,611)	Processing protease	± (12.5)	+ (280)
albF	427 (48,979)	Ubiquinol-cytochrome *c* reductase complex, Zn endoproteinase (N terminal only)	− (<0.5)	+ (280)
albG	234 (26,333)	? (membrane protein)	± (25)	+ (280)

[a] Length is in amino acids, and molecular mass is in daltons.
[b] Percentages of wild-type activity, based on arbitrary units per microgram of cellular protein, are shown in parentheses.
[c] Defect in immunity observed in anaerobic incubation. MICs were measured under anaerobic conditions.
[d] Value is for the *sboA*::*neo-2* mutant.

A ***albB* complementation**



B **Immunity to subtilosin**

Strain	Chloramphenicol addition	MIC subtilosin
JH642	NA	282
ORB3400 (*albB*::*cat*)	-C	45.3
	+C	63.2
ORB 3472 (*sboAXalbABC'* / *albB*::*cat*)	-C	96.5
	+C	233.5

FIG. 3. Complementation of the *albB* mutation by the *amyE*::*sboAXalbABC'* construct. (A) Genomic organization of *albB*/*amyE*::*sboAXalbABC'*. The circle indicates the *B. subtilis* genomic map, and the locations of the replication origin (*oriC*) and termination site (*ter*) are shown. The *amyE* and *sbo-alb* loci are labeled on the genomic map, and the organization of the *sbo-alb* operon bearing the *albB*::*cat* mutation and the *sbo-alb* DNA of the *amyE*::*sboAXalbABC'* locus is shown. (B) MICs of subtilosin for the wild-type parent, JH642, the *albB*::*cat* mutant ORB3400, and the *albB*::*cat*/*albB* diploid ORB3472. An immunity assay was performed on anaerobically grown lawns of cells on 2×YT medium supplemented with glucose and KNO_3 as described in Materials and Methods. The assay was performed in the presence (+C) or absence (−C) of chloramphenicol. NA, not applicable.

drives expression of downstream *alb* genes, results in nearly complete complementation of the *albB* mutation. This confirms that the *albB* gene product is required for immunity and also indicates that the expression of *albC* and *-D* is needed for complete immunity.

The *sboA*::*neo-1* insertion mutant contains a *neo* gene cassette within the *sboA* coding sequence. The *neo* gene is transcriptionally oriented in the same direction as the *sbo-alb* operon (49) and has been shown to direct high-level expression of the downstream *alb* genes. Accordingly, the *sboA*::*neo-1* mutant shows levels of subtilosin immunity higher than that exhibited by wild-type cells (MIC, >560 μg/ml). This higher level of self-protection can be attributed to the enhanced expression of *albBCD* in the *sboA*::*neo-1* mutant. (Note that the value in Table 3 is that of the *sboA*::*neo-2* mutant, in which the *neo* gene is oriented oppositely to the *sbo-alb* direction of transcription.)

Identification of the *sboX* gene and detection of its expression in anaerobic cultures. Inspection of the *sboA* and *sboA-albA* intergenic region revealed an open reading frame that could potentially encode a bacteriocin-like product (Fig. 4). The coding sequence of this putative gene, *sboX*, begins at the 30th codon of *sboA* and extends to 28 nucleotides upstream from the TTG start of *albA*. A putative ribosome-binding site lies 16 to 20 bp from the TTG start codon. The product of *sboX* resembles a precursor of a type II bacteriocin in that it possesses a putative GG cleavage processing site, is cationic, and bears a hydropathy profile similar to that of some carnobacteriocins (32). To determine if the putative product of *sboX* functions in the production of antilisterial activity, an insertion mutation was introduced into the *sboX* open reading frame. Two restriction sites, *Hin*dIII and *Bam*HI, were constructed in the sequence corresponding to the 16th and 17th codons, respectively, of the *sboX* coding sequence. The *Hin*dIII site was used to create a translation *lacZ* fusion as described below. Into the *Bam*HI site was inserted a neomycin resistance cassette that would result in the insertional inactivation of the *sboX* gene. Introduction of the insertion into the *sboX* gene of the JH642 chromosome resulted in reduction of subtilosin activity, as shown by incubation of *sboX*::*neo* mutant colonies in a lawn of *L. monocytogenes* cells (data not shown). The *neo* cassette used was the same as that used to create *sboA*::*neo-1*, which was shown to direct the transcription of the downstream *alb* genes (49). It is unlikely that the insertion exerts a negative polar effect on *alb* gene expression.

An in-frame deletion mutation was created that removed a segment of the *sboX* coding sequence extending from bp 89 to bp 138, replacing the fragment with a *Bam*HI site (Fig. 4B). The resulting allele, *sboXΔ89-139*, did not confer a subtilosin-negative phenotype, nor did it detectably affect subtilosin production or immunity. It also did not have an effect on the expression of an ectopically expressed *sboA-lacZ* fusion (49). We conclude that the putative *sboX* product does not function in subtilosin production and is not required for antilisterial activity. The *sboX*::*neo* insertion mutations resides within the stem-loop structure located at the end of the *sboA* gene. It is possible that the insertion results in lower subtilosin production because it confers instability on the *sboA* mRNA.

To determine if the *sboX* coding sequence was translated in *B. subtilis*, the *Hin*dIII site was used to fuse *sboX* in frame with the truncated *lacZ* gene of plasmid ptrpGB1. The construct was introduced into the *amyE* locus, and *lacZ* activity was measured in samples collected from anaerobic and aerobic cultures. No *sboX-lacZ* expression could be detected in cells of aerobic cultures (Fig. 4C), but induction of expression was observed as anaerobic cultures entered the stationary phase of growth.

DISCUSSION

Eight of the nine genes of the *sbo-alb* operon function in the production of the antilisterial bacteriocin subtilosin, as shown by the phenotype produced by insertion mutations. Of the mutations created, those in *sboA*, *albA*, and *albF* are the most critical for the formation of active subtilosin. Mutations in *sboX*, *albB*, *albC*, *albD*, *albE*, and *albG* do not abolish subtilosin production but impair antilisterial activity. We do not know the primary functions of the *alb* gene products. The polypeptides encoded by *albA*, *-C*, *-E*, and *-F* show primary structural similarity to known proteins. AlbC very likely functions in the export of subtilosin, which it performs, perhaps, in conjunction with other *alb* products. We propose that AlbA, very likely a member of the MoeA/NifB/PqqE family (16), and AlbF, a member of a family of zinc endoproteinases, perform critical modifications of the presubtilosin peptide. AlbB, -C, and -D are required for immunity to subtilosin.

The newly discovered *sboX* gene encodes a bacteriocin-like precursor peptide bearing a GG motif resembling type II prebacteriocin cleavage sites (27). A *neo* cassette insertion muta-

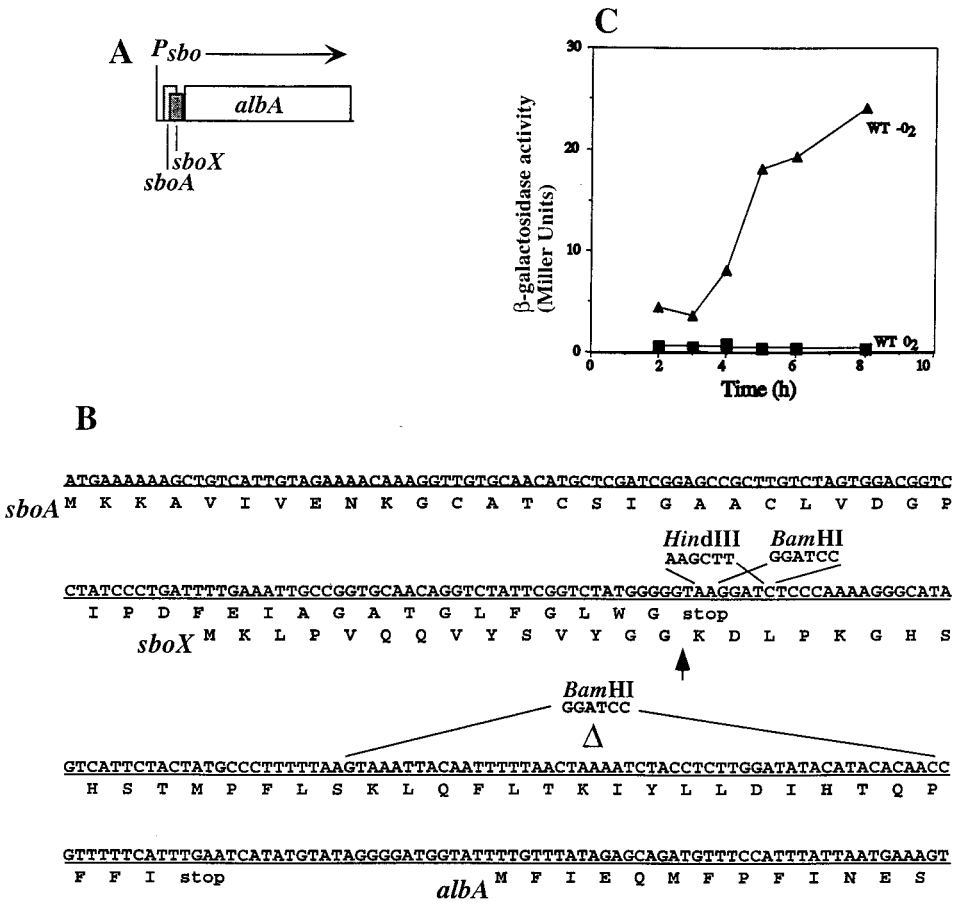


FIG. 4. Organization of the *sboAX* locus. (A) Diagram of the *sboA*, *sboX*, and *albA* region of the *sbo-alb* gene cluster. P*sbo* indicates the location of the *sbo-alb* operon promoter, and the arrow marks the direction of transcription. (B) Nucleotide sequence of the *sboAX* genes and amino acid sequence (below the horizontal line) of the products. The restriction enzyme sites introduced into the *sboX* coding sequence (*Hin*dIII and *Bam*HI) are indicated. The segment of the *sboX* gene that is deleted in the *sboXΔ89-139* allele is labeled Δ. The *Bam*HI sequence that replaced this segment is indicated. (C) Expression of *sboX*::*lacZ* under anaerobic ($-O_2$) and aerobic (O_2) conditions. An *sboX-lacZ* translational fusion was introduced into the *amyE* locus of *B. subtilis* JH642 cells. WT, wild type.

tion in *sboX* interrupts its coding sequence but also damages the sequence encoding the stem-loop structure residing at the 3′ end of *sboA*. The insertion would eliminate the *sboX* product but might also render *sboA* mRNA unstable and susceptible to 3′ exonucleolytic activity, which could explain the reduced subtilosin production of the *sboX*::*neo* mutant. The in-frame deletion allele *sboXΔ89-139* has no detectable effect on subtilosin production or immunity or regulation of *sboA* expression. We do not know the function of the *sboX* gene.

AlbA and other members of the MoaA/NifB/PqqE family (16) possess two Cys clusters, one in the N-terminal half of the protein and the other at the C terminus. These are thought to be the locations of Fe-S centers that serve as the active sites in reactions involving hydration or dehydration of substrate compounds (38, 45). MoaA catalyzes a step of molybdenum cofactor synthesis in which precursor Z is produced from the phosphorylated guanosine precursor prior to MoeB-dependent sulfur addition (30, 45). Other members function in the synthesis of enzyme cofactors such as pyrroloquinoline quinone (5, 17, 43) and siroheme (11). A homolog of AlbA is encoded by the *ybcQ* gene of *B. subtilis*, which appears to reside in an operon that contains a small gene, *ybcO*, encoding another bacteriocin-like product (15).

The amino acid sequence of the N-terminal half of AlbF is very similar to those of known beta chain Zn metalloproteases of mitochondria that are associated with the cytochrome *Bc1* reductase complex (3, 9, 29, 31) and the product of *pqqF*, which functions in cofactor (pyrroloquinoline quinone) synthesis (17, 44). The C-terminal half of AlbF shows no homology to known proteins but exhibits some similarity to the peptide binding protein encoded by *oppA* of *Myxococcus xanthus*. We suspect that AlbF catalyzes some step in the processing of presubtilosin.

Mutations in *albB*, *-C*, *-D*, and *-G* do not abolish subtilosin production but appear to reduce the amount of active peptide produced. In most cases, we do not know if partially active processing intermediates are produced and secreted or if the mutants simply produce less wild-type product. In the case of the *albG* mutant, nearly wild-type levels of a peptide are found in the culture supernatant, as judged from LH-20 chromatography and Tricine SDS-PAGE, but the activity of the product is significantly reduced (data not shown). This could be due to the accumulation of a processing intermediate that is efficiently secreted into the medium. The structure of this peptide product is under investigation.

Mutations in *albB*, *-C*, and, to a lesser extent, *-D* reduced the cell's immunity to subtilosin. The product of *albC*, a member of the ABC family of transport proteins, very likely participates in export of subtilosin. In several bacteriocin and lantibiotic production systems, export proteins have been shown to be re-

quired for complete immunity to the specific peptide produced (33, 35). The small 59-amino-acid hydrophobic peptide encoded by *albB* appears to play a critical role in immunity, since the *albB* mutant shows the most severe defect in subtilosin self-protection. AlbB may serve the same function as the other small, hydrophobic immunity peptides encoded by lantibiotic biosynthesis operons. Like other genes that confer bacteriocin immunity, *albB* is required for maximal bacteriocin production. How these products function in conferring self-protection and in bacteriocin production is not known.

In a previous report, we presented evidence that one or more products of the *alb* genes may function in the positive autoregulation of *sbo-alb* expression (49). This was based on the observation that high constitutive expression of the *alb* genes results in accelerated expression of an ectopically expressed *sboA-lacZ* fusion. We examined the effect of each *alb* insertion mutation on *sboA-lacZ* expression but found no significant changes. The high-level expression of the *alb* genes probably does not play a direct role in *sbo-alb* transcriptional regulation.

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